# PATENT COOPERATION TREATY

# PCT

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)





| Applicant's or agent's file reference<br>67306-71242 | **FOR FURTHER ACTION** | See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416) |
|---|---|---|
| International application No.<br>PCT/SE 03/00547 | International filing date *(day/month/year)*<br>04.04.2003 | Priority date *(day/month/year)*<br>04.04.2002 |
| International Patent Classification (IPC) or both national classification and IPC<br>C12Q1/68 | | |
| Applicant<br>BIOTAGE et al. | | |

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 5 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the opinion
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

| Date of submission of the demand | Date of completion of this report |
|---|---|
| 26.09.2003 | 14.06.2004 |
| Name and mailing address of the international preliminary examining authority:<br>European Patent Office<br>D-80298 Munich<br>Tel. +49 89 2399 - 0 Tx: 523656 epmu d<br>Fax: +49 89 2399 - 4465 | Authorized Officer<br>Knudsen, H<br>Telephone No. +49 89 2399-8696 |

# INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/SE 03/00547

## I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:

**Description, Pages**

| | |
|---|---|
| 1-29, 31, 33 | as originally filed |
| 30, 32 | filed with telefax on 28.04.2004 |

**Claims, Numbers**

| | |
|---|---|
| 1-18 | filed with telefax on 28.04.2004 |

**Drawings, Sheets**

| | |
|---|---|
| 1/9-9/9 | as originally filed |

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

   - ☐ the description, pages:
   - ☐ the claims, Nos.:
   - ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)).

   *(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)*

6. Additional observations, if necessary:

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

| | | | |
|---|---|---|---|
| Novelty (N) | Yes: | Claims | 1-18 |
| | No: | Claims | |
| Inventive step (IS) | Yes: | Claims | 1-18 |
| | No: | Claims | |
| Industrial applicability (IA) | Yes: | Claims | 1-18 |
| | No: | Claims | |

2. Citations and explanations

   **see separate sheet**

**Re Item V**

**Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

Reference is made to the following documents:

D1: WO-A2-0194546

D2: WO-A2-0053812

NOVELTY:

None of the cited documents disclose a method with the features of present claim 1 and claims 1-16 are therefore considered novel.

In addition, none of the cited documents disclose a kit with the components indicated in claim 17 and claims 17-18 are therefore considered novel.

INVENTIVE STEP:

The closest prior art document for claim 1 is D2 (example 17) which discloses a method for primer extension employing a mix of 10mM Tris (pH=7.5), 50mM NaCl, 5mM $MgCl_2$, 0.1 mg/ml BSA, 0.01% Triton X-100,0. 0.1mM unlabelled dCTP and 0.2 µM Cy5-SS-dCTP and incubation for 4 minutes at room temperature.

The method of claim 1 differs from this method in that the labelled nucleotide is present in an molar ratio of 1-50 mole %, whereas in D2 the labelled nucleotide is present in a ratio of 67 mole %.

According to the present application's description, the problem solved by reducing the amount of labelled nucleotide is that the signal reflects better the number of nucleotides incorporated. because quenching or formation of disulfide bridges within the extended primer which occurs in D2 is reduced.

The solution to the problem of providing a method with an improved signal linearity is the reduction of the amount of labelled nucleotide.

The prior art D1 teaches the use of mixtures for primer extension which contains between 30 and 95 mole % unlabelled nucleotide, the reason given in D1 for using this ratio of unlabelled species is that an increased reliability of the incorporation of nucleotides when using thermostable polymerase for the extension method is observed.

However, none of the cited documents provide any hint of the possibility of solving the problem discussed above and shown to be solved in examples 2 and 3, in a method in which the label is attached to the nucleotide by a cleavable linker. Claim 1 is therefore considered inventive. Claims 2-16 are considered inventive for the same reasons.

The only document which concerns a method in which a cleavable linker is used in order to improve the precision of the claimed method is D2. Thus, D2 is the only document based on which the skilled person would contemplate incorporating a reducing agent in a kit. However, as explained above, D2 uses a higher percentage of labelled nucleotides and therefore does not suggest a kit as claimed in claim 17. Thus, present claims 17-18 are considered inventive.

D1 (claim 39) discloses a composition comprising a mixture of labelled and unlabelled forms of a nucleotide wherein the unlabelled form is 30 to 95% of the combined amount of the amounts on a molar basis. Moreover, D1 (claims 44-50) discloses a kit comprising the mixture of labelled and unlabelled forms of a nucleotide. However, D1 does not suggest the use of a cleavable linker or reducing agent and claims 17-18 are therefore inventive over D1 as well. For the reasons given above for claim 1, the skilled person would not combine D1 and D2 and thereby arrive at the claimed kit.

INDUSTRIAL APPLICABILITY:

Present claims 1-18 are considered industrially applicable.

The mixes contained 20% Cy5-SS-dCTP, 30% Cy5-SS-dATP or 30% Cy5-SS-dGTP with the balance made up with the corresponding natural deoxynucleotide.

As can be seen in Figure 4, the signals obtained were reproducible and stable throughout the sequence for the different nucleotides. The internal variation in signal height between different bases was due to differences in the way Klenow exo$^-$ polymerase accepts the labeled nucleotides. The level of incorporation of nucleotides was checked by analyzing the immobilized templates by pyrosequencing using PSQ 96 and associated kits according to the manufacturers instructions (Pyrosequencing AB, Sweden) such that the absence of a peak at the point of dispensing respective dNTPs was indication of complete incorporation in the foregoing experiment. All incubations gave better than 95% incorporation as assessed by pyrosequencing (results not shown).

Example 5: Determining the selectivity of Klenow exo- DNA polymerase for labeled/unlabelled nucleotides

E3PN10B

Fluorescein-GTAAAACGACGGCCAGT**C**
CAACATTTTGCTGCCGGTCAGACTTGCTTAAGGTCG-biotin

E3PN4B

Fluorescein-GTAAAACGACGGCCAGT**A**
CAACATTTTGCTGCCGGTCATGCTGCTTAAGGTCG-biotin

E3PN12b

the Cy5-labelled nucleotide (excitation 590 nm, emission 670 nm) and the fluorescence of the fluorescein-labeled primer (excitation 485nm, emission 535 nm) using a fluorimeter (Victor2, Perkin-Elmer). The fluorescein signal was used to normalize results for variation in transfer of beads.

The level of incorporation of nucleotides was checked by analyzing the immobilized templates by pyrosequencing using PSQ 96 and associated kits according to the manufacturers instructions (Pyrosequencing AB, Sweden) such that the absence of a peak at the point of dispensing the relevant nucleotide was indication of complete incorporation in the foregoing experiment. All incubations gave better than 95% incorporation as assessed by pyrosequencing (results not shown).

The results in Figures 5-8 show the selectivity of the polymerase for labeled against non-labeled nucleotides. There are clear differences in how the polymerase accepts the different Cy5-SS-nucleotides, in particular between U* and G*.

Example 6: Determining the relationship between fluorescence signal and number of bases incorporated in homopolymer stretches with Cy5-SS-dGTP/dGTP mixes according to WO 00/53812

E3PN13b: 5′-GCTGGAATTCGTATGCACTGGCCGTCGTTTTACAAC-3′

E3PN24b: 5′-GCTGGAATTCGTATGCCACTGGCCGTCGTTTTACAAC-3′

E3PN25b: 5′-GCTGGAATTCGTATGCCCACTGGCCGTCGTTTTACAAC-3′

NUSPT-FL: 5′-Fluorescein-GTA AAA CGA CGG CCA GT-3′

## Claims

1) A method for determining the sequence of a nucleic acid molecule comprising the steps of;

a) providing a single-stranded form of said nucleic acid molecule;

b) hybridizing a primer to said single stranded form of said nucleic acid molecule to form a template/primer complex;

c) enzymatically extending the primer by the addition of a polymerase and a mixture of at least one nucleotide and at least one labeled derivative of the at least one nucleotide, wherein the at least one labeled derivative of the at least one nucleotide comprises a label linked to the nucleotide via a cleavable link and wherein the amount of labeled derivative of the at least one nucleotide in said mixture of the at least one nucleotide and the labeled derivative of the at least one nucleotide is within the range of 1-50 mole-%, 1-40 mole-%, 1-30 mole-%, or 1-20 mole-%.

d) determining the type of nucleotide added to the primer; and

e) repeating steps c) to d) at least once.

2) A method according to claim 1, in which the amount of labelled derivative of the at least one nucleotide in said mixture is within the range of 5-50 mole-%, 5-40 mole-%, 5-30 mole-%, or 5-20 mole-%.

3) A method according to claim 1, in which the amount of labelled derivative of the at least one nucleotide in said mixture is within the range of 10-50 mole-%, 10-40 mole-%, 10-30 mole-%, or 10-20 mole-%.

4) A method according to any one of claims 1 – 3, wherein the single-stranded form of said nucleic acid molecule is attached to a carrier.

5) A method according to claim 4, wherein the the means for attachment is selected from the group of: a) specific binding to a hydrophobic compound, an oligonucleotide, an antibody or a fragment thereof, a protein, a peptide,

an intercalating agent, biotin, streptavidin or avidin; or b) covalent coupling using an amino-linker and an epoxy-treated carrier.

6) A method according to claim 4, wherein the carrier is selected from the group of a gel, a solid or porous bead, a surface or a fiber.

7) A method according to any one of claims 1 - 3, in which the label is neutralized after step d) by the addition of a label-interacting agent or by bleaching.

8) A method according to claim 7, in which the bleaching is performed by photo-bleaching.

9) A method according to claim 1-3 in which the link between the incorporated nucleotide and the label is cleaved after step d).

10) A method according to claim 1-3, in which the link between the fluorophore and nucleotide is a disulfide bond.

11) A method according to claim 10 in which the cleavage is performed by the addition of a reducing agent, thereby exposing a thiol group.

12) A method according to claims 10 or 11, in which the exposed thiol group is capped by a suitable reagent, such as iodoacetamide or N-ethylmaleimide.

13) A method according to any of the claims above in which the linker between the disulfide bridge and the base is shorter than 8 atoms.

14) A method according to any of the above claims in which the step c) is performed at a pH below 7, preferably at a pH below 6.5, or more preferably at a pH below 6.

15) A method according to any of the above claims, in which the derivative of said nucleotide is a dideoxynucleotide or an acyclic nucleotide analog.

16) A method according to any of the above claims, in which an agent chosen from the group comprising the following; alkaline phosphatase, PPi-ase, apyrase, dimethylsulfoxide, polyethylene glycol, polyvinylpyrollidone, spermidine, detergents such as NP-40, Tween 20 and Triton X-100; various proteins that affect secondary structure of DNA including Single Stranded DNA Binding Protein (SSB) or the protein of Gene 32, is added.

17) A mixture of at least one nucleotide and at least one labelled derivative of the at least one nucleotide, wherein the at least one labeled derivative of the at least one nucleotide comprises a label linked to the nucleotide via a cleavable link and wherein the amount of labeled derivative of the at least one nucleotide in said mixture of the at least one nucleotide and the labeled derivative of the at least one nucleotide is within the range of 1-50 mole-%, 1-40 mole-%, 1-30 mole-%, or 1-20 mole-%, preferably in the range of 5-20 mole-%, 5-30 mole-%, 5-40 mole-% or 5-50 mole-%, and even more preferably in the range of 10-20 mole-%, 10-30 mole-%, 10-40 mole-% or 10-50 mole-%.

18) A kit comprising, in separate compartments, a mixture according to claim 17, and at least one of the following components; a DNA polymerase, a reducing agent, a carrier, a capping agent, an apyrase, an alkaline phosphatase, a PPi-ase, a single strand binding protein or the protein of Gene 32, for performing the method according to any of the claims 1-14.